American Journal of Hematology 61:187–193 (1999)

Hb Iraq-Halabja β10 (A7) Ala→Val (GCC→GTC): A New β-Chain Silent Variant in a Family with Multiple Hb Disorders

S. Deutsch,[1] R. Darbellay,[1] R. Offord,[2] A. Frutiger,[3] J. Kister,[4] H. Wajcman,[5] and *Ph. Beris[1]

[1]Division d'Hématologie, Hôpital Cantonal Universitaire de Genève, Switzerland
[2]Département de Biochimie médicale, CMU, Université de Genève, Switzerland
[3]Laboratoire de Chimie Clinique, Hôpital Cantonal Universitaire de Genève, Switzerland
[4]INSERM, U473. Le Kremlin—Bicêtre, France
[5]INSERM, U468. Le Kremlin—Bicêtre, France

A patient originating from Iraq was referred to our laboratory upon suspicion of a hemoglobinopathy. Routine hematological tests revealed a microcytic and slightly anemic phenotype with an elevated HbA_2 suggestive of β-thalassemia. Samples were obtained for several members of the family which upon examination revealed highly heterogeneous phenotypes that prompted us to investigate the case further. Sequencing of the β-globin gene and α cluster mapping in the propositus and his brother showed a previously undescribed β-globin variant:Hb Iraq-Halabja, β10(A7) Ala→Val (GCC→GTC), associated with β^0-thalassemia IVS-2 nt1 G→A and either α-thal-2-3.7 kb deletion (brother), or α-globin gene triplication anti-3.7 kb type (propositus). Detailed functional studies of the variant gave a normal oxygenation curve, a normal heterotopic action of 2,3 DPG, and normal heat stability and isopropanol precipitation tests. The variant shows a clear difference in migration properties compared to normal β-chain only when run on PAGE urea Triton. As expected, α/β-globin mRNA ratios were influenced by the concomitant presence of an α-globin gene pathology and the β^0 thalassemia and not by the presence of the β-globin variant which apparently is clinically silent. Am. J. Hematol. 61:187–193, 1999.

Key words: β-thalassemia; hemoglobin variant; α/β mRNA ratios; hemoglobin modeling; α-globin triplication

INTRODUCTION

About 700 different hemoglobin (Hb) variants have been described in the literature [1]. Some of them cause alterations in the physiology of the protein affecting parameters such as oxygen affinity or protein stability, producing a range of hematological abnormalities including polycythemia, cyanosis, and hemolysis. However, the majority of Hb variants that have been observed in human populations do not affect the functioning of the protein and are thus clinically silent.

The study of Hb variants has played a fundamental role in helping to define the structure/function relationships of this complex protein [2]. The detailed knowledge of the structure of Hb and the recent development of accurate three-dimensional computer models has permitted explanations of the phenotypes (or their absence) associated with many variant structures to be put forward.

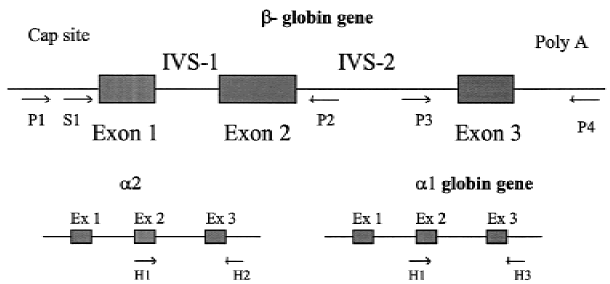
In this paper, we describe a new β-globin variant found in two Iraqi patients and affecting codon 10 of the peptide, where an alanine residue is replaced by a valine. Interestingly, 3 other globin abnormalities (β^0-thalassemia, α-thalassemia type 2, and α-globin triplication) were found within the same family, thus providing

Contract grant sponsor: Swiss National Foundation for Scientific Research to PB.

Contract grant number: 32-31342.91.

*Correspondence to: P. Beris, M.D., Division d'hématologie, Hôpital Cantonal Universitaire de Genève, CH - 1211 GE 14, Switzerland. E-mail: Photis.Beris@hcuge.ch

Received for publication 12 October 1998; Accepted 3 March 1999



Primer	Sequence
P1	5' GCCAAGGACAGGTACGGCTGTCATC
P2	3' CCCTTCCTATGACATGAACTTAACCAT
P3	5' TATCATGCCTCTTTGCACCATTC
P4	3' AATGCACTGACCTCCCACATTCC
S1	5' ACACAACTGTGTTCACTAGC
H1	5' GGCAAGAAGGTGGCCGACG (α1, α2)
H2	5' GGGAGGCCCÂTCGGGCAGGAGGAA (α2)
H3	5' GGGGGGAGGCCCAAGGGGCAAGAA (α1)
H4	5' CCTGTGGGGCAAGGTGAA (β Exon 1)
H5	5'GAGCCAGGCCATCACTAAAG (β Exon 2)

Fig. 1. Amplification and sequencing primers. Note that specific reverse primers for each α gene were used for convenience because all validation studies were performed on the combined primer set, although for this work we did not study α gene-specific expression.

the opportunity to study the interactions between the different mutations, and the effect that each of these has on the patients' phenotypes.

MATERIALS AND METHODS

The propositus was a 36-year-old man originating from Halabja in north Iraq, who presented microcytosis and slight to moderate anemia. Hematological studies revealed thalassaemic phenotype, which was later found to be present in other members of his family. It is worth mentioning that his son (#5829) was found to suffer from moderate to severe anemia, which motivated further studies.

Blood samples were collected in vacucontainers with EDTA as anticoagulant. Full hematological data were obtained with an automated hematological analyzer (Sysmex NE 8000) and routine laboratory procedures (serum iron, serum transferrin, serum ferritin). DNA was extracted from peripheral blood by standard methods [3]. HbA_2 was quantified by microcolumn chromatography by using diethylaminoethyl cellulose (DEAE), and HbF was quantified by using alkali denaturation, as described in previous papers [4]. Isoelectric focusing (IEF) was performed by using the Resolve Ampholyte pH 6–8 gel according to the manufacturer's instructions (Isolab, Akron, OH). PAGE on Urea-Triton was performed as described in Alter et al. [5].

Specific primers (P1–P4, see Fig. 1) for the β-globin gene were used for 30 cycles of PCR amplification (94°C 1 min, 60°C 1 min, 72°C 1 min) in a mixture containing 500 nM of each primer, 200 nM of DNTPs and 1.5 mM $MgCl_2$ added to a standard QIAGEN buffer. In this reaction, one of the amplification primers was 5′ biotinylated [6] and, under these conditions, single-stranded template DNA was prepared by using magnetic beads (Dynabeads M-280 streptavidin) and a magnetic particle concentrator (Dynol MPC-E UK). Direct sequencing of the isolated product was performed by using appropriate internal sequencing primers and following previously described technology of Sanger et al. [7] based on the sequenase enzyme and dideoxy chain termination method with some modification.

N-terminal microsequencing of the protein was performed in order to confirm the molecular biology findings. For this, 10 μg of α and β-globins extracted from a hemolysate were loaded on a 15% mini SDS gel and run under reducing conditions. Electroblotting onto PVDF membranes and amidoblack staining was done as described by Hughes et al. [8]. N-terminal sequence determination was performed by using a model 473 A microsequencer (Applied Biosystems, Foster City, CA) equipped with a Problott reaction cartridge.

α-cluster gene mapping was performed to detect the possible presence of deletional α thalassemia type 1 or 2, which are by far the most common forms. For this, genomic DNA was digested with a number of restriction enzymes (*Eco*R1, *Xba*I, and *Bgl*II) and hybridized to specific probes in a Southern blotting reaction as previously described [9,10]. mRNA was extracted from blood according to the method of Chomczynski and Saachi [11] and diluted to a concentration of 100 ng/l. A routine RT reaction was then performed (using less than 500 ng of RNA as starting material) for 1 hr by using M-mlv reverse transcriptase (Life Technologies). The cDNA products were amplified for 17-21 cycles in a multiplex PCR reaction by using fluorescently labeled primers for the α and β-globin genes (H1–H5, Fig. 1). Different fluorochromes were used for each gene [12]. The α to β mRNA ratios were quantified by using laser-induced fluorescence, and detected on a standard automated sequencer. Analysis of the relative intensities was carried out using the Gene Scan fragment analyzer software (Perkin–Elmer).

For mRNA ratio determination to be valid using an end-point reverse transcription polymerase chain reaction (RT-PCR)-based method, the primers involved must have a very similar amplification efficiency and all measurements must be taken during the exponential phase of the reaction. Provided these conditions are met (see below), it is convenient to perform multiplex PCR reactions, because the PCR products can then be directly compared, without the use of an internal control.

The validity of our protocol was confirmed first by quantifying the α/β DNA ratio in a series of control subjects. Because it is known that individuals without globin gene abnormalities have two α-globin genes and

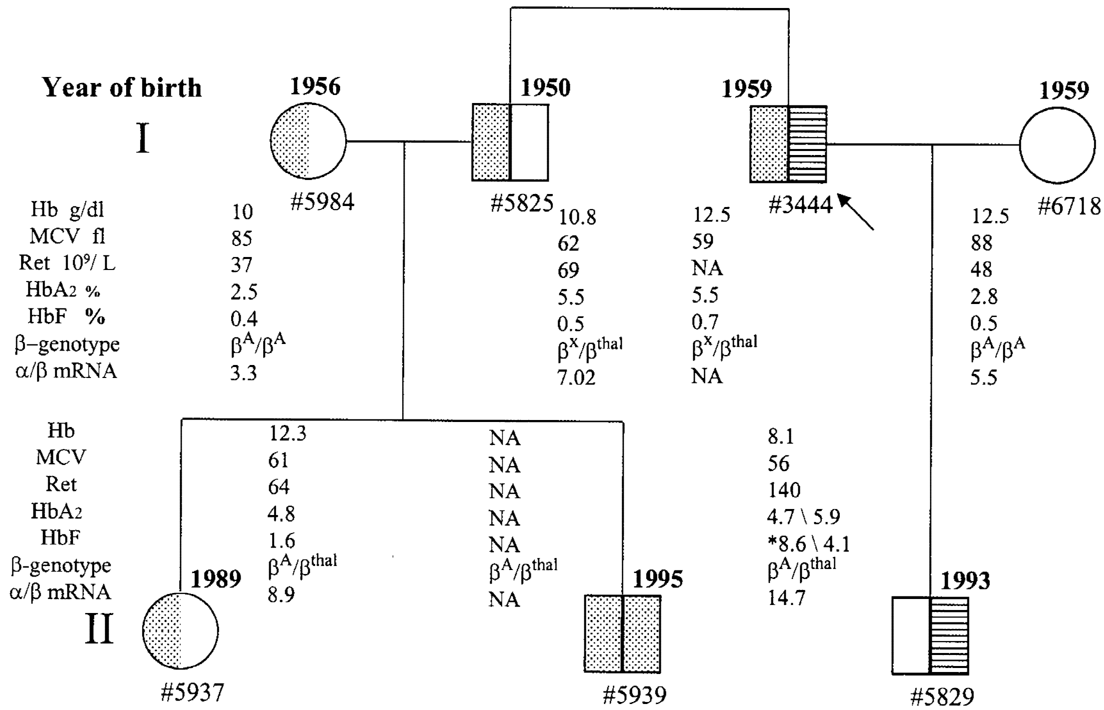


Fig. 2. Pedigree of family showing hematological data, α and β genotypes, and mRNA ratios. Shaded area = α-thalassemia type 2, 3.7 kb deletion; striped areas = α-globin triplication anti-3.7 type. *Value at 2 years old.

one β-globin gene in each chromosome, we would expect to see a ratio of 2. We observed in practice an average ratio of 2.3. In addition, we amplified globin cDNAs in a series of control subjects, and took aliquots from single PCR reactions at different cycles [17–21]. We measured the α to β ratios from these samples and found little variation (variance = 1.3). However, after cycle 21 the ratios had a tendency to decrease, indicating that the reaction was reaching the plateau phase.

These two validation experiments showed that the efficiencies of amplification of the primer sets used was very similar, and that the PCR range at which the samples were studied was still within the exponential phase of the reaction.

Heat stability and isopropanol precipitation tests were performed by standard methods [13] to detect the possible presence of abnormal hemoglobin. Oxygen equilibrium curves were performed on fresh blood cell suspensions from patient #5825 by using an automatic continuous method (Hemox-Analyzer, TCS Medical Products), as previously described [14].

For solution studies, 50 mM EDTA and 29 mg/ml catalase were added in order to limit methemoglobin formation. P_{50} and n_{50} values were calculated by linear regression from the Hill equation for oxygen saturation levels between 40% and 60%.

Theoretical atomic coordinates for the molecular structure of the mutant β-chain were calculated by using the SWISS-MODEL system [15–17], accessible through the Expasy Molecular Biology Server of the Swiss Institute for Bioinformatics (http://www.expasy.ch). The template used for the calculation was a molecular coordinate set for the β-chain of the structure of deoxy Hb A, refined at 1.74 Å resolution [18], Brookhaven Protein Data Bank code 2HHB. Illustrations for this paper were prepared using version 2.6 of the RASMOL software developed by Dr. Roger Sayle. RASMOL was also used for distance calculations.

RESULTS

Figure 2 gives the pedigree of the family members studied, their hematological data and the results of gene mapping of the α cluster.

The propositus #3444 and his brother #5825 present microcytic anemia with high Hb A_2. The morphology of the blood smear was typical for β-thalassemia in the heterozygous state (data not shown).

Children #5937 and #5829 carry also the β-thalassemia trait, with patient #5829 presenting a more severe anemia with hemolytic features and high Hb F. The sample was analysed in 1998, when child #5829 was 5 years old.

The wives of both brothers (#5984 and #6718) have normal hematological parameters except a slight anemia in subject #5984. No hematological data were available for patient #5939 because molecular biology studies were done on cord blood.

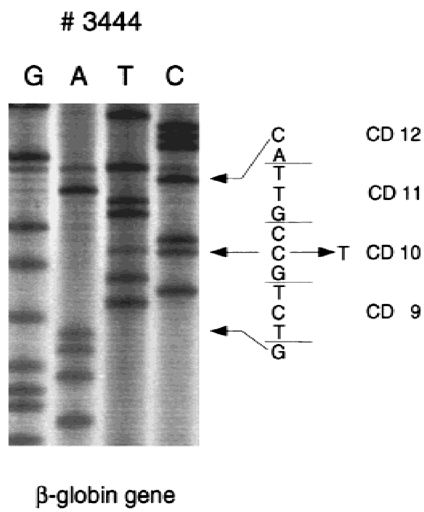


Fig. 3. Sequencing data for a region of β-globin exon 1 in patient #3444. A double band at codon 10 can be observed indicating a C→T mutation (heterozygous state) at this site that had not been previously described.

Figure 3 gives sequencing data concerning exon 1 of the β-globin gene in patient #3444, which shows the presence of a GCC to GTC mutation in codon 10. This mutation had not been previously reported, and was also found to be present in patient #5825 (as shown in Fig. 2). Sequencing of the exon 2 - IVS 2 junction from the same patient (#3444) showed the presence of a known G→A mutation at position IVS-2-1 [19], which leads to a β^0 thalassaemic phenotype. This mutation was found in the heterozygous state in many members of the family (#5825, #5937, #5939 and #5829) and explains the β thalassemia features initially observed.

Although we do not have a patient homozygous for the codon β10 mutation, material recovered from patient #5825, who carries this mutation in the heterozygous state, was eminently suited for protein studies, because the other gene copy has the β^0 thalassaemic mutation, allowing a homogenous sample of the mutant form to be obtained. N-terminal microsequencing of the α- and β-chains from this person confirmed the molecular biology findings, because only a Val residue was found at position β10 (data not shown), with no sign of the Ala which occurs at this position of the normal chain.

In functional studies performed on patient #5825, fresh red blood cell suspensions gave a normal oxygenation curve, with a P_{50} value of 27 mmHg and a Hill coefficient n_{50} of 2.5 in standard buffer (pH 7.4 Bis-Tris 0.05 M, NaCl 0.14 M at 37°C). Equally, the equilibrium oxygenation curve on the stripped hemolysate, containing almost pure Hb Iraq-Halabja, was identical to that of normal Hb A (P_{50} = 5.2 mmHg, n_{50} = 2.7 at pH 7.2, Bis-Tris 0.05 M, NaCl 0.1 M. 25°C). The heterotropic action of 2,3 DPG was also found to be normal with a P_{50} value of 16 mmHg and n_{50} value of 2.8 in the presence of 1 mM DPG.

Heat stability and isopropanol precipitation tests done with the hemolysate of patient #5825 gave negative results, and migration of the variant hemoglobin on PAGE gave normal results ($\beta^{\text{Iraq-Halabja}}$-chain urea pH 9.0 = 20, $\beta^{\text{Iraq-Halabja}}$-chain urea pH 6.0 = 20, identical to normal β-chain) except when run on PAGE urea Triton where a clear difference in the migration properties was observed ($\beta^{\text{Iraq-Halabja}}$-chain urea triton = 21.9, normal β-chain = 20) (Fig. 4).

Results from the mRNA quantification experiments are also given in Fig. 2.

DISCUSSION

We describe a new β-globin variant at position β10 (A7) in which there is an Ala to Val substitution. The variant was first reported by us in an abstract in 1995 [20]. We do not have this variant in the simple heterozygous state, but its presence in association with a β^0 mutation allowed us to obtain the variant protein in a pure form, ideal for functional studies. The results of these tests showed no instability or modification in the oxygenation curves that would suggest a functional anomaly. Furthermore, hematological data of patients #5825 and #5937 were shown to be similar, in both cases they presented the β^0 mutation in association with the thalassemia type 2, 3.7 kb deletion, with the codon β10 mutation only present in the former patient.

Position β10 is an external residue and, as shown in Figure 5, it is distant from the heme, the β interface, and the β- β crevice. Although it is therefore likely that the mutation will be clinically silent, this is not an absolute guarantee. To cite but one example, Hb Shuangfeng (Glu α27→Lys) is unstable [21], but α27 is a surface residue. The mutation at β10 could have an effect if it significantly moved or distorted helix A, in which it lies. Given that the mutation involves an increase in the size of the side-chain, it is important to know if there is enough room between β10 on the one hand, and Val β126 and Val β129, because these residues, situated on the H helix, are spatially quite close. Calculation (not shown) indicates that there is sufficient room to accommodate the enlarged side-chain, and so the A helix will not be displaced by the mutation. Modelling shows that the helix is not distorted either. Figure 6 illustrates the fact that, as expected, the lower helix propensity of the valine relative to that of the alanine [22] does not seem to perturb the A helix in the mutant. This is reminiscent of the situation with Hb Ankara [23], in which β10 is aspartic acid, a residue similar in its helix propensity to valine:Hb Ankara is associated with an apparently silent phenotype.

All this is in contrast to, for example, the neighboring

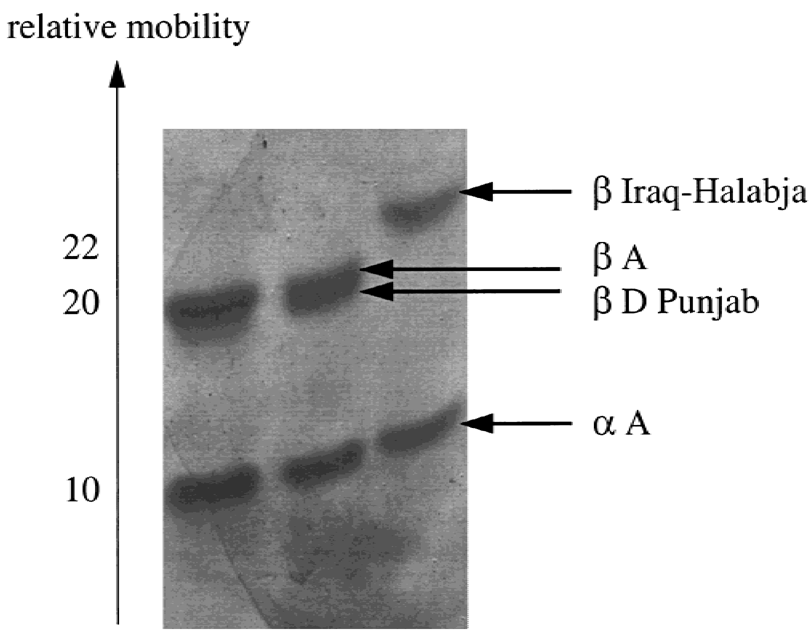


Fig. 4. Polyacrylamide gel electrophoresis of globin in 6 M urea in the presence of Triton X-100. From left to right: normal control, patient heterozygous for Hb D Punjab, patient compound heterozygous for β thalassemia and Hb Iraq-Halabja.

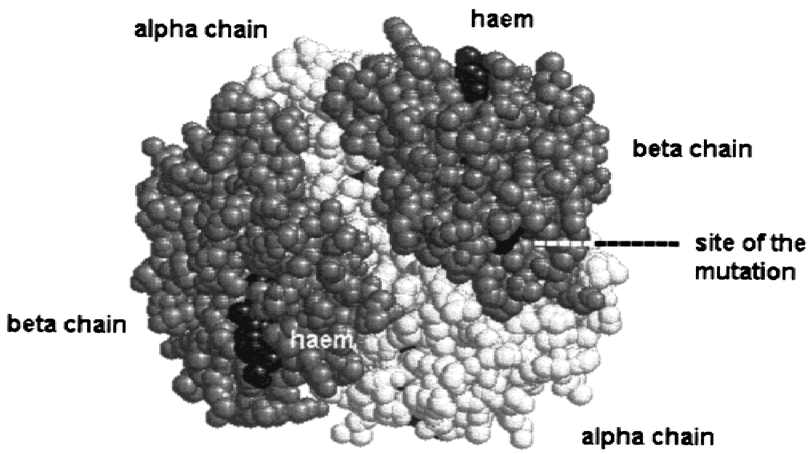


Fig. 5. A general view of deoxyhemoglobin A. In this view, the β-chains are in front (shaded subunits) and the α-chains behind (white subunits), The heme groups are shown in black, as is the site of the codon-10 mutation on one of the β-chains. The site on the other β-chain is obscured by the bulk of the subunit.

residue, β11. This is an internal residue, and of the three known variants, only the one with a highly conservative change, from Val to Ile, is without phenotypic consequences.

The abstract possibility always exists that a mutation that increases the bulk and hydrophobicity could by chance find a complementary hydrophobic pit in a neighboring tetramer and give rise to a sickling phenomenon. While such a possibility is hard to eliminate by theoretical modeling, the side-chain of residue β10 protrudes markedly less from the surface of the β subunit than does the side-chain of residue β6, the site of the classical sickling mutation. In the event, we observed no phenotypic consequences of the mutation whatsoever.

As the new variant was described in a family that originated from Halabja in north Iraq, this hemoglobin was designated Hb IRAQ-HALABJA. It is interesting to mention that a high proportion of the population of this region, including our family, is of Kurdish origin, an ethnic group in whom other mutations have been described [24]. Different associations of β-thal, α-thal type 2 and α-globin triplication were found in this family. As expected, the association of β-thal with the α α α/ α α genotype created a more severe picture, with hemolytic features, also reflected in the impressive alterations of red blood cells in the peripheral blood smear (see also Fig. 7). The clinical differences between #5937 and #5829 are due to the difference in α gene status: β^A/β^{thal}; $-\alpha^{3.7}/\alpha\ \alpha$ versus β^A/β^{thal}; $\alpha\ \alpha\ \alpha^{anti\text{-}3.7}/\alpha\alpha$.

This is in accordance with other cases that have been published [25], and is a consequence of the high α/β imbalance leading to hemolysis, caused by the excess α-chains that precipitate, and can be observed as inclusion bodies. This imbalance is clearly shown in the excessively high α/β mRNA ratio in patient #5829 shown in Figure 2.

All other ratios were as expected, with increases relative to the normal value in patients with the β^0 mutation,

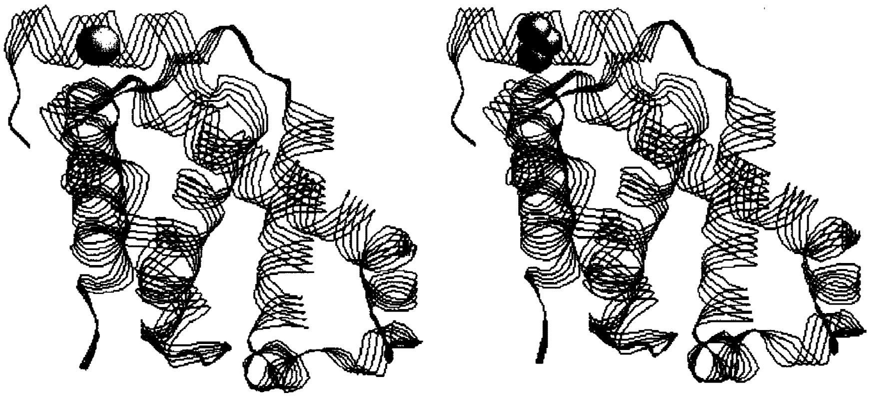


Fig. 6. Views of the β-chains of the normal deoxy Hb A (left) and of the mutant form (right) from a position side-on to the A helix. The side-chain carbon atoms at residue 10 are shown as spheres.

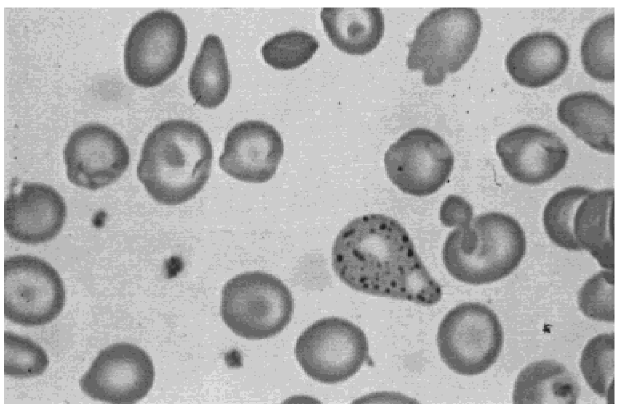


Fig. 7. Peripheral blood smear of patient #5829.

and a modest but significant decrease in the ratio in patient #5984 in whom only the α-thal type 2 deletion was present.

The absolute value of these ratios is slightly higher than those previously published [12]; however these differences are not surprising if one considers that the detection methods used are completely different.

An interesting point to mention is that patient # 5937 has a significantly higher α/β ratio than patient #5825, although initially we had expected both of these patients to have very similar values. A possible explanation to account for some of this difference, is that patient #5937 shows an elevated Hb F (1.6%) value not present in patient #5825, indicating that a proportion of the reticulocyte population from this patient would be expressing γ-globin together with α-globin (and no β-globin), hence increasing the ratio between the two adult globin chains.

In conclusion, we describe a new β-globin silent variant in an Iraqi family (Ala β10→Val) which is associated with β^0-thal, α-thal type 2, -3.7 kb deletion and α-globin triplication anti-3.7 kb type. These hemoglobin abnormalities are present in different combinations in the members of this family, accounting for the heterogeneity in the hematological picture that was initially observed.

ACKNOWLEDGMENTS

We thank Jean Riou and Hedwige Trachsel for excellent technical assistance.

REFERENCES

1. Huisman THJ, Caver MFH, Efremov GD, eds. A syllabus of human hemoglobin variants. Augusta, GA: The Sickle Cell Anemia Foundation: 1996.
2. Perutz MF. Molecular anatomy, physiology, and pathology of hemoglobin. In: Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus P, editors. The molecular basis of blood disorders. Saunders: Toronto; 1987, pp. 127–162.
3. Poncz M, Solowlejczyz D, Harpel B, Mory Y, Schawtz E, Surrey S. Construction of human gene libraries from small amounts of peripheral blood. Analysis of β-like globin genes. Hemoglobin 1982;6:27.
4. Mach-Pascual S, Darbellay R, Pilotto P-A, Beris P. Investigation of microcytosis: a comprehensive approach. Eur J Haematol 1996;57:54.
5. Alter BP, Goff SC, Efremov GD, Gravely ME, Huisman THJ. Globin chain electrophoresis: a new approach to the determination of the γ/α ratio in fetal haemoglobin and to studies of globin synthesis. Br J Haematol 1980;44:527.
6. Hultman T, Stahl S, Hornes E, Uhlen M. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. Nucleic Acids Res 1989;17:4937.
7. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Nat Acad Sci USA 1977;74:5463.
8. Hughes GJ, Frutiger S, Paquet N, Ravier F, Pasquali Ch, Sanchez J-C, James R, Tissot D, Bjellqvist B, Hochstrasser D. Plasma protein map: an update by microsequencing. Electrophoresis 1992;13:707.
9. Felice AE, Cleek MP, Mckie K, Mckie V, Huisman THJ. The rare α-thalassemia of blacks is a ζ-α-thalassemia-1 associated with deletion of all α and ζ globin genes. Blood 1984;63:1253.
10. Beris P, Grossiord D, Hochmann A, Zhu L, Miescher P-A. Dèpistage de l'α-thalassémie par analyse du DNA. Schweiz. Med. Wschr 1998; 118:1538.
11. Chomczynski P, Saachi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Analyt Biochem 1987;1162:156.
12. Smetania NS, Leonova JY, Levy N, Huisman THJ. The α/β and α1/α2 ratios in different forms of α-thalassemia. Biochim Biophys Acta 1996;1315:188.
13. Kim HC, Schwartz E. Unstable Hemoglobins. In Beutler E, Lichtman MA, Coller BS, Kipps TJ, editors. Williams Hematology. Fifth edition, New York: McGraw Hill, 1990, pp. L33–L34.
14. Kister J, Poyart C, Edelstein SJ. An expanded two-state allosteric

model for interactions of human hemoglobin A with nonsaturating concentrations of 2,3-diphosphoglycerate. J Biol Chem 1987;262: 12085.

15. Peitsch MC. Protein modelling by E-mail. Bio-Technology 1995;13: 658.
16. Peitsch MC. ProMod and the Swiss-Model: Internet-based tools for automated comparative protein modelling. Biochem Soc Trans 1996; 24:274.
17. Guex N, Peitsch MC. SWISS-MODEL and the Swiss Pdb viewer: an environment for comparative protein modelling. Electrophoresis 1997; 18:2714.
18. Fermi G, Perutz MF, Shaanan B, Fourme R. The cystal structure of human deoxyhaemoglobin at 1.74 Å resolution. J Mol Biol 1984;175: 159.
19. Treisman R, Proudfoot NJ, Shander M, Maniatis T. A single base change at a splice site in a β^{0}-thalassemic gene causes abnormal RNA splicing. Cell 1982;29:903.
20. Beris P, Darbellay R, Frutiger A, Hochstrasser D. Hb Iraq-Halabja 10 (a7) Ala→Val (G$\underline{C}$C→G$\underline{T}$C): a new β-chain variant that produces no significant clinical or hematological alterations. Blood 1995;86(suppl 1):642A.
21. Chihchuan L, Hainan T, Hweiyuen L, Shangzhi H, Ruiyou L, Banseng W . Hemoglobin Shuangfeng (α 27 (β8) Glu substituting for Lys): a new unstable hemoglobin variant. Hemoglobin 1981;5:691.
22. Chakrabartty A Baldwin RL . Stability of helices. Adv Prot Chem 1995;46:141.
23. Harano T, Harano K, Ueda S, Shibata S, Imai K. Hemoglobin Ankara [β10(A7) Ala→Asp]: properties and biosynthesis. Hemoglobin 1981; 5:737.
24. Kazazian HH, Boehm, CD. Molecular basis and prenatal diagnosis of β-thalassemia. Blood 1988;72:1107.
25. Beris P, Darbellay R, Hochmann A, Pradervand E, Pugin P. Interaction of the heterozygous β^{0}-thalassemia and triplicated α globin loci in a Swiss-Spanish family. Klin Wschr 1991;69:710.